Calcium-Dependent Conformation of Desmoglein 1 Is Required for its Cleavage by Exfoliative Toxin

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In bullous impetigo, *Staphylococcus aureus* spreads under the stratum corneum of skin by elaboration of exfoliative toxin, which hydrolyzes only one peptide bond in a highly structured calcium-binding domain of desmoglein 1, resulting in loss of its function. We investigated the basis of this exquisite specificity. Exfoliative toxin cannot cleave desmoglein 1 pretreated at 56°C or higher or at low or high pH, suggesting that the proper conformation of desmoglein 1 is critical for its cleavage. Because cleavage occurs in an area of desmoglein 1 stabilized by calcium, we determined if the conformation necessary for cleavage is calcium-dependent. Depletion of calcium from desmoglein 1 completely inhibited its cleavage by exfoliative toxin, even after calcium was added back. A change in conformation of desmoglein 1 by calcium depletion was shown, with immunofluorescence and enzyme-linked immunoassay, by loss of binding of PF sera, which recognize conformational epitopes. This change in conformation was confirmed by tryptophan fluorometry and circular dichroism, and was irreversible with repletion of calcium. These data suggest that the specificity of exfoliative toxin cleavage of desmoglein 1 resides not only in simple amino acid sequences but also in its calcium-dependent conformation. ***Key words: cadherins/impetigo/serine protease/Staphylococcus. J Invest Dermatol 121:383–389, 2003***

Impetigo is the most common bacterial infection of children and 30% of these impetigo patients have bullous impetigo, which is caused by *Staphylococcus aureus* strains that produce exfoliative toxins (ETs) (Ladhani *et al*, 1999). Staphylococcal scalded skin syndrome is a generalized form of bullous impetigo in which patients, usually infants or young children, develop bullae and erosions over a large area of the skin surface due to the systemic circulation of ETs from a localized source of infection. In the early 1970s, two serotypes of ETs (ETA and ETB), were shown to produce blisters in the superficial epidermis of neonatal mice by passive transfer. (Melish and Glasgow, 1970, 1971; Melish *et al*, 1972; Kondo *et al*, 1974). Subsequently, a third ET (ETD), which also causes blisters in neonatal mice, was isolated and characterized (Yamaguchi *et al*, 2002). Recently, it has been shown that these ETs are serine proteases that specifically target and cleave desmoglein (Dsg) 1 (Amagai *et al*, 2000, 2002; Yamaguchi *et al*, 2002). The exquisite specificity of this protease–substrate interaction is underscored by the finding of one unique cleavage site caused by all ETs in both mouse and human Dsg1, and the inability of ETs to cleave closely homologous proteins such as Dsg3 and E-cadherin (Amagai *et al*, 2000; Hanakawa *et al*, 2002).

Dsg1 is a member of the cadherin supergene family (Angst *et al*, 2001). These cadherins characteristically contain extracellular repeating domains of about 100 amino acids in length that are highly conserved. Most cadherins are involved in calcium-dependent cell–cell adhesion, and calcium is thought to be important in maintaining their structure and function (Kemler *et al*, 1989; Steinberg and McNutt, 1999).

Dsg1 is a member of the so-called desmosomal cadherin family of molecules which includes desmogleins and desmocollins. These molecules are critical to the proper function of desmosomes that maintain tissue integrity in epithelial and other tissues (Green and Gaudry, 2000). The importance of these molecules in maintaining adhesion in epithelial tissues has been demonstrated by loss of adhesion with anti-desmoglein and anti-desmocollin antibodies from patients with pemphigus, cell culture studies in which these desmosomal cadherins confer cell–cell adhesion, and loss of adhesion in mice with genetic deletions of genes encoding these molecules (Chitaev and Troyanovsky, 1997; Hashimoto *et al*, 1997; Koch *et al*, 1997a; Marcozzi *et al*, 1998; Mahoney *et al*, 1999; Yasuda *et al*, 2000; Chidgey *et al*, 2001). As with classical cadherins, calcium is critical to the function of desmosomal cadherins, and the amino acid sequence of desmosomal and classical cadherins is highly conserved at the calcium-binding sites (Garrod *et al*, 1996; Chitaev and Troyanovsky, 1997; Wallis *et al*, 2000; Syed *et al*, 2002). Therefore, like classical cadherins, calcium is presumed to maintain the structure and function of desmosomal cadherins.

ETs have been shown to attack Dsg1 precisely at one of its calcium-binding domains. They hydrolyze the peptide bond just after the glutamic acid at amino acid position 381 (as counted from the initiating methionine of both mouse and human Dsg1), between extracellular domains 3 and 4. By homology to the recent crystal structure of C-cadherin this cleavage site is in the third of four calcium-binding sites each of which binds three calcium ions (Boggon *et al*, 2002). These sites are thought to orient rigidly each extracellular domain to the next to result in a rod-like structure of the entire extracellular domain. Thus ETs show exquisite specificity for a highly structured site in Dsg1. This led us to ask whether this specificity resides only in the primary amino acid sequence of Dsg1 or in its conformation, and, if

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Abbreviations: ETs, exfoliative toxins.

the latter, whether ETs conformational specificity was calcium dependent.

MATERIALS AND METHODS

Recombinant ETs Recombinant wild-type ETA and ETB with a V5 and His tag on the carboxy-terminus (these tagged ETs will be abbreviated ETA and ETB) were purified on Ni-NTA columns (Qiagen, Valencia, California) according to the manufacturer's protocol, then dialyzed against phosphate-buffered saline (Hanakawa *et al*, 2002). Protein concentrations of ETA and ETB were estimated with a Protein Assay Kit (Bio-Rad Laboratories, Hercules, California).

Production, pretreatment and ET cleavage of Dsg1 The entire extracellular domain of human recombinant Dsg1 with a his and E-tag (hDsg1E) on the carboxyl-terminus was produced as a secreted protein by baculovirus as previously described (Ishii *et al*, 1997). Approximately, 10 nM of hDsg1E in the culture supernatant of High Five (Ca^{2+} concentration of 6 mM) was treated with various conditions then used for cleavage analysis as follows: (1) hDsg1E was incubated at 4, 25, 56, and 80°C for 1 h, then incubated with 3 μM ETA at 37°C for 1 h. (2) hDsg1E was dialyzed against Tris-buffered saline (TBS) with 1 mM $CaCl_2$ (TBS + Ca^{2+}), then the pH was adjusted to 4.0 and 4.5 by adding 0.1 M acetic acid, pH 4.0 and 4.5, which was preadjusted with NaOH. pH was adjusted to 5.0 and 6.0 by adding 0.1 M MES (2-(N-Morpholino)ethanesulfonic acid) buffer, pH 5.0 and 6.0, which was preadjusted with NaOH. pH was adjusted to 7.4 with 0.1 M Tris of pH 7.4. pH was adjusted to 10 with 0.1 M NaOH. After incubation at room temperature for 15 min, the solutions were dialyzed against TBS + Ca, then incubated with 3 μM ETA at 37°C for 1 h. (3) hDsg1E in culture supernatant was dialyzed against TBS + Ca^{2+}, then treated with 5 mM ethylenediamine tetraacetic acid (EDTA) at room temperature for 1 h, then again dialyzed against TBS + Ca^{2+}. As a control, hDsg1E dialyzed against TBS + Ca^{2+} without pretreatment with EDTA was used. Dialyzed hDsg1E was then incubated with 3 μM ETA or, as controls, 3 μM porcine pancreas trypsin (Sigma, St Louis, Missouri) or 3 μM staphylococcus V8 protease (Roche Applied Science, Indianapolis, Indiana) at 37°C for 1 h. (4) hDsg1E was dialyzed against TBS without $CaCl_2$ ($TBS_{minus}Ca^{2+}$), TBS + Ca^{2+} or $TBS_{minus}Ca^{2+}$ followed by TBS + Ca^{2+}, then incubated with 3 μM ETA or ETB at 37°C for 1 h. In all the above four conditions, degradation of hDsg1E was assayed by western blotting with anti-E-tag antibodies.

For fluorometry and circular dichroism analysis, hDsg1E was purified from culture supernatant with an anti-E-tag column (Amersham Biosciences, Piscataway, New Jersey) and eluted using 0.1 mg per mL E-tag peptide in TBS + Ca^{2+} and 0.1% octylglucoside (Anatrace, Maumee, Ohio). Eluant was dialyzed against TBS + Ca^{2+} and 0.1% octylglucoside for fluorometry, and water with 1 mM $CaCl_2$ for circular dichroism analysis. The purified samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then stained by Coomassie Brilliant Blue, which showed one major band (**Fig 1**). Protein concentrations of hDsg1E were estimated with a Protein Assay Kit (Bio-Rad Laboratories).

Western blotting Proteins in Laemmli sample buffer were separated by 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories or Invitrogen Life Sciences, Carlsbad, California), then transferred to nitrocellulose sheets (Transblot; Bio-Rad Laboratories). The sheets were incubated for 1 h in blocking buffer of 5% fat-free milk powder in phosphate-buffered saline. The E-tag antibody conjugated with horseradish peroxidase (Amersham Biosciences), diluted in blocking buffer, was applied for 1 h at room temperature. After four washes with 0.1% Tween 20 in phosphate-buffered saline, the signals were detected with chemiluminescence (ECL or ECL plus, Amersham Biosciences).

Immunofluorescent staining A pemphigus foliaceus (PF) patient's serum (PF982) was diluted to 1:1280 with TBS + Ca^{2+} or with 2 nM hDsg1E, which was purified on an anti-E tag column and dialyzed against $TBS_{minus}Ca^{2+}$ or TBS + Ca^{2+}, then incubated overnight at 4°C. These diluted sera were used for indirect immunofluorescent staining of cryostat sections of normal human skin. Epidermal bound human IgG from the diluted pemphigus sera were detected with antihuman IgG conjugated with Alexa 590 (Molecular Probes, Eugene, Oregon).

Enzyme-linked immunosorbent assay (ELISA) hDsg1E was purified with the anti-E-tag column, then dialyzed against TBS + Ca^{2+} (hDsg1E + Ca^{2+}) or $TBS_{minus}Ca^{2+}$ (hDsg1E$_{minus}Ca^{2+}$). PF sera (n = 8), normal human serum (n = 2), or bullous pemphigoid serum (n = 2) were diluted into various concentrations of hDsg1E + Ca^{2+} or hDsg1E$_{minus}Ca^{2+}$ and incubated overnight at 4°C.

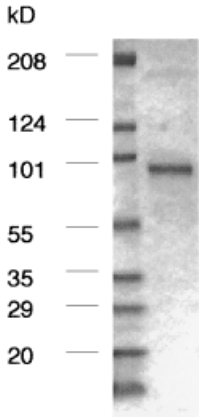

Figure 1. Purified hDsg1E. Purified hDsg1E were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, then stained by Coomassie Brilliant Blue, which showed one major band at 84 kDa.

For ELISA substrate, Ni-NTA HisSorb strips (Qiagen) were coated by incubation with 2 nM hDsg1E + Ca in TBS + Ca^{2+} with 1% bovine serum albumin, overnight at 4°C. Normal, bullous pemphigoid, or PF sera, adsorbed with hDsg1E + Ca^{2+} or hDsg1E$_{minus}Ca^{2+}$, were used as the first antibody. Anti-human IgG conjugated with alkaline phosphatase (DAKO, Carpinteria, CA) was used as the second antibody. The plates were developed with AmpliQ kit (DAKO) according to the manufacturer's procedure and the OD at 490 nm was measured with an ELISA plate reader.

Circular dichroism Circular dichroism measurements were performed with an AVIV Model 202 spectrometer. Far-ultraviolet spectra from 200 nm to 250 nm were recorded of hDsg1E solution at 25°C in a quartz cell with a path length of 1 mm. The analysis was of hDsg1E at a concentration of 5 μM in water with 1 mM $CaCl_2$, pH approximately 6.0. For the second spectrum EDTA was added to a final concentration of 5 mM. Finally, the third analysis was taken following further addition of excess Ca^{2+} (final concentration 10 mM $CaCl_2$). Spectra were corrected by subtraction of each corresponding solution, and data were normalized and presented as mean molar residue ellipticity (deg cm^2 per dmol) using the calculation of:

$$[\theta] = \frac{(\text{CD value of protein} - \text{CD value of buffer})}{(10 \times \text{conc in } M \times \text{path length in cm} \times \#\ \text{residues})}$$

Three independent preparations of hDsg1E, two in water and one in phosphate-buffered saline, pH 7.4, showed similar spectra.

Fluorometry Tryptophan fluorescence emission of hDsg1E was measured with a QM-C60 fluorescence spectrophotometer (Photon Technology, Lawrenceville, NJ) in the photon counting mode. The cell holder was maintained at 25°C. Excitation was at 295 nm, and intrinsic fluorescence emission spectra were recorded from 305 to 405 nm at a rate of 4 nm per s and at 1 nm intervals. Excitation and emission slits were set to give a 4 nm band pass, and the cuvette had a path length of 1 cm. Background spectra of buffer were subtracted from all hDsg1E spectra to correct for the Raman fluorescence. In EDTA experiments, spectra of the same hDsg1E were taken in the following sequence: (1) in TBS + Ca^{2+}, (2) following addition of EDTA to a final concentration of 5 mM, then (3) following further addition of excess Ca^{2+} (to 10 mM $CaCl_2$). In pH experiments, spectra of the same hDsg1E were taken in the following sequence: (1) in TBS + Ca^{2+}, (2) following addition of 4×10^{-4} M HCl to yield a pH of 3.5, then (3) following further addition of 10 mM Tris pH 7.4 to adjust the pH to 7.0.

Finally, spectra were taken at 1 and 13 min after hDsg1E was directly added to 8 M urea in TBS + Ca^{2+}.

RESULTS

ETA does not cleave heat-denatured Dsg1 To determine, in general, if the conformation might be important in Dsg1 cleavage by ET, we analyzed if heating of Dsg1 affects its proteolysis (**Fig 2*A***). Recombinant Dsg1E (extracellular domain of human Dsg1 with an E-tag at the carboxy-terminus) produced by baculovirus was treated at 4, 25, 56, or 80°C for 1 h, then incubated with excess ETA at 37° for 1 h. hDsg1E and its degradation product were detected by western blotting with anti-E-tag antibody. hDsg1E pretreated at 4°, 25° or 37°C was cleaved by ETA to yield the characteristic 35 kDa degradation product, but ETA was not able to cleave hDsg1E heated at 56° or 80°C. In contrast, trypsin, a serine protease in the same family as ETA, was able cleave hDsg1E after pretreatment at all temperatures (data not shown). Trypsin and other related proteases in this family (the chymotrypsin family) are sequence-dependent proteases and can cleave denatured proteins. These results suggest that heat may irreversibly change the conformation of Dsg1, and ETA cleavage of Dsg1 may be dependent on conformation, not just primary amino acid sequence.

Dsg1 pretreated at low and high pH was not cleaved by ETA We next determined whether changes in pH, which may change the conformation of proteins, could affect the susceptibility of Dsg1 to proteolysis by ETA. hDsg1E was treated at pH 4.0, 4.5, 5.0, 6.0, and 7.4 for 15 min at room temperature then dialyzed against TBS + Ca^{2+} (pH 7.4) before incubation with ETA. Subsequent western blotting with anti-E-tag antibody indicated hDsg1E treated at pH 4.5 and below lost its susceptibility to proteolysis by ETA (**Fig 2*B***). hDsg1E treated with high pH (pH 10 with NaOH) was also not cleaved by ETA (data not shown). These data again indicate that Dsg1 susceptibility to cleavage by ETA is not simply specified by its primary amino acid sequence, but is likely conformational dependent. In addition, these findings imply that any changes induced in conformation of Dsg1 by pH are not directly reversible by reversing the pH back to 7.4, as confirmed below by tryptophan fluorescence spectroscopy (**Fig 6**).

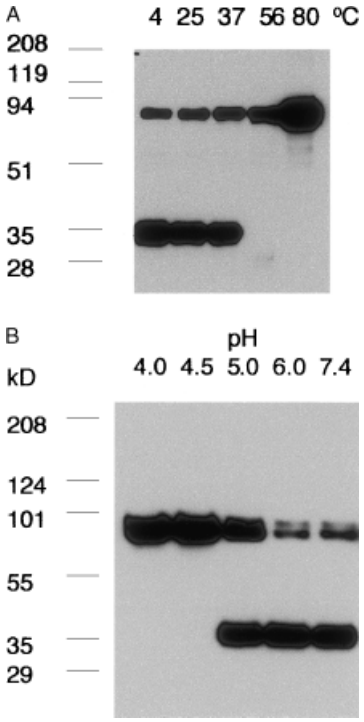

Figure 2. The effect of heat and pH on Dsg1 susceptibility to cleavage by ETA. (*A*) After incubation at 4°C, 25°C, 37°C, 56°C, and 80°C for 1 h, hDsg1E was incubated with ETA for 1 h at 37°C. Western blotting with anti-E-tag antibodies showed that hDsg1E preincubated at 56°C and 80°C were not cleaved by ETA. (*B*) After treatment with pH 4.0, 4.5, 5.0, 6.0, and 7.4, hDsg1E was dialyzed against TBS + Ca^{2+} (pH 7.4) and incubated with ETA for 1 h. at 37°C. Western blotting with anti-E-tag antibody showed that hDsg1E pretreated at pH 6.0 and 7.4 was cleaved by ETA but pretreatment at pH 4.0 and 4.5, inhibited almost all cleavage. hDsg1E pretreated at pH 5.0 showed partial cleavage.

Ca^{2+}-depleted Dsg1 was not cleaved by ET and sequential addition of Ca^{2+} did not reverse susceptibility to proteolysis The Ca^{2+} binding in cadherins is mediated by acidic amino acids, which have negative charge at neutral pH (Ringwald *et al*, 1987; Kemler *et al*, 1989; Ozawa *et al*, 1990; Nagar *et al*, 1996; Pertz *et al*, 1999; Boggon *et al*, 2002). Therefore, low pH would be likely to cause loss of Ca^{2+} binding, and this loss may partially explain how low pH denatures Dsg1. To investigate directly whether proteolysis of Dsg1 by ET is dependent on the Ca^{2+}-stabilized structure of Dsg1, we incubated hDsg1E with EDTA, and then dialyzed it against TBS + Ca^{2+} before incubation with ETA (**Fig 3*A***). As controls for ETA we used trypsin and staphylococcus V8 protease, which are well-characterized sequence-specific serine proteases in the same family (chymotrypsin family) as ETA. hDsg1E not treated with EDTA was cleaved by ETA to yield the characteristic carboxy-terminal 35 kDa degradation product. Trypsin and V8 protease also digested Dsg1, but, as expected, no degradation product was visible on western blotting, because the substrate is digested into small peptides or alternatively, it is possible that these proteases cleaved off the E-tag. EDTA did not block hDsg1E digestion by trypsin and V8 protease, but did inhibit cleavage by ETA, even after Ca^{2+} was added back. Similarly, ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA)-treated Dsg1 was no longer susceptible to degradation by ETA (data not shown). Finally, as with ETA, ETB could not cleave EDTA or EGTA-treated Dsg1 even after Ca^{2+} was added back (data not shown).

We then determined whether dialysis alone, without a chelating reagent, could affect the susceptibility of Dsg1 to proteolysis by ETs (**Fig 3*B***). When hDsg1E was dialyzed against $TBS_{minus}Ca^{2+}$, it was not cleaved by ETA or ETB. When hDsg1E was $TBS_{minus}Ca^{2+}$ and sequentially dialyzed against TBS + Ca^{2+}, it was still mostly resistant to degradation by ETA and ETB, although a slight amount of cleavage was observed. hDsg1E dialyzed against TBS with 1 mM $MgCl_2$ and TBS with 1 mM $ZnCl_2$ was not cleaved by ETA and ETB (data not shown).

These results indicate that the cleavage of Dsg1 by ET is dependent on a Ca^{2+}–Dsg1 interaction, and once Ca^{2+} is removed, Dsg1 loss of susceptibility to cleavage is almost completely irreversible. These data, combined with the known Ca^{2+}-dependent conformation of cadherins, suggest that depletion of Ca^{2+} cause at least partial denaturation of Dsg1 that is not totally reversible once Ca^{2+} is restored. Combined with the loss of susceptibility to proteolysis by ET after heat and pH changes, these data suggest that the exquisite specificity of ET for Dsg1 is dependent on the conformation of Dsg1 that is stabilized by Ca^{2+}.

Change in conformation of Dsg1 with loss of Ca^{2+} demonstrated by PF autoantibodies and ELISA assay To investigate Ca^{2+}-dependent changes in conformation of Dsg1, we measured its reactivity with PF sera, as it has been shown that most antibodies from PF sera bind conformational epitopes on Dsg1 (Koulu *et al*, 1984; Stanley *et al*, 1986; Eyre and Stanley, 1987; Amagai *et al*, 1995a, b; Kowalczyk *et al*, 1995) (**Fig 4*A***). IgG in PF sera bind the cell surface of keratinocytes throughout the epidermis as determined by indirect immunofluorescence. hDsg1E stabilized with Ca^{2+} was able to adsorb anti-

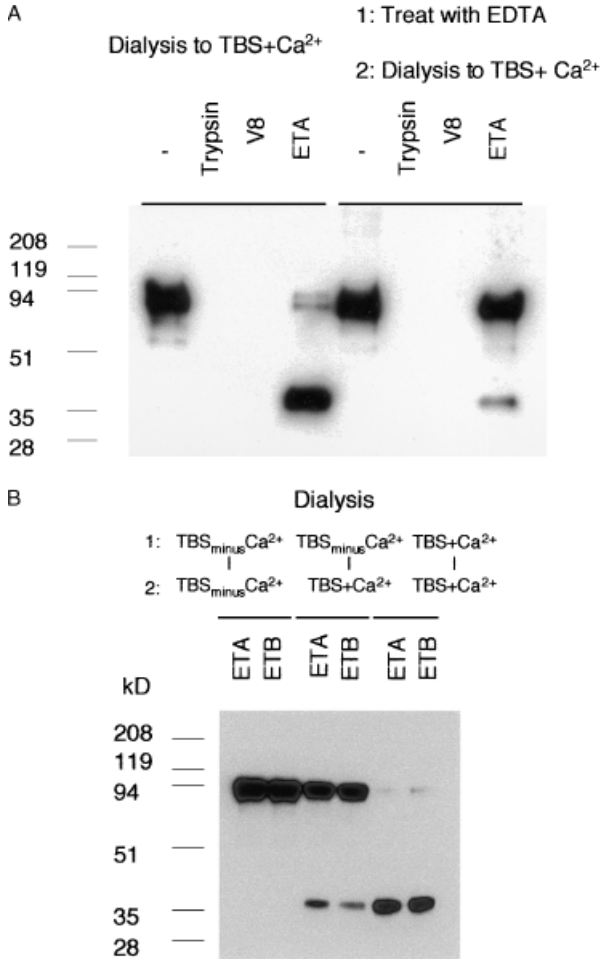

Figure 3. Loss of Ca^{2+} renders Dsg1 resistant to cleavage by ETA and ETB even after Ca^{2+} is added back. (*A*) hDsg1E was pretreated with buffer or EDTA, then dialyzed against TBS + Ca before incubation with ETA, trypsin, or staphylococcus V8 protease. Western blotting with anti-E-tag antibody showed that hDsg1E was not degraded by ETA if it was pretreated with EDTA, but was degraded by the other proteases. (*B*) Before incubation with ETA or ETB, hDsg1E was sequentially dialyzed against $TBS_{minus}Ca^{2+}$ then again against $TBS_{minus}Ca^{2+}$; $TBS_{minus}Ca^{2+}$ then against $TBS + Ca^{2+}$; and $TBS + Ca^{2+}$ then again against $TBS + Ca^{2+}$. Dsg1 dialyzed against $TBS_{minus}Ca^{2+}$ was not cleaved, Dsg1 dialyzed against $TBS_{minus}Ca^{2+}$ then $TBS + Ca^{2+}$ was only slightly cleaved, compared with hDsg1E dialyzed against $TBS + Ca^{2+}$, which was fully cleaved.

keratinocyte antibodies from PF sera; however, strikingly, in contrast, hDsg1E dialyzed against $TBS_{minus}Ca^{2+}$ did not adsorb most of these cell-surface reactive antibodies.

To semiquantitate these findings, we performed ELISA assays on Dsg1 using PF sera adsorbed with $hDsg1E + Ca^{2+}$ or $Dsg1E_{minus}Ca^{2+}$ (**Fig 4*B***). Normal human serum (n = 2) and bullous pemphigoid serum (n = 2) showed background reactivity to Dsg1 and pretreatment with $Dsg1E_{minus}Ca^{2+}$ or $Dsg1E + Ca^{2+}$ did not cause a significant change in their reactivity. On the other hand, PF sera adsorbed with $hDsg1E_{minus}Ca^{2+}$ showed high reactivity with Dsg1 that was mostly, although not entirely in some sera, adsorbed with $hDsg1E + Ca^{2+}$. These data show that Dsg1 undergoes a major conformational change when Ca^{2+} is removed, and that this change removes most of the epitopes that bind anti-Dsg1 autoantibodies in PF sera.

Analysis of the Ca^{2+}-dependent conformation of Dsg1 by circular dichroism and tryptophan fluorometry To investigate the Ca^{2+}-dependent change in conformation of Dsg1 and its potential reversibility more directly, we performed circular dichroism and tryptophan fluorometry, two techniques that measure parameters dependent on a molecule's conformation.

In circular dichroism (**Fig 5**), the spectrum of hDsg1E in 1 mM $CaCl_2$ showed a minimum of -8.35×10^3 deg cm^2 per dmol at 215 nm. The shape of the spectrum suggested the predominance of β sheets, consistent with the crystal structure of the ectodomains of cadherins (Shapiro *et al*, 1995; Nagar *et al*, 1996; Tamura *et al*, 1998; Pertz *et al*, 1999; Boggon *et al*, 2002). After Ca^{2+} depletion with EDTA, a decrease of amplitude with a left shift of the minimum was observed, as has been shown for E-cadherin (Pokutta *et al*, 1994). This change is consistent with a gross change in the structure of Dsg1 after removal of Ca^{2+}. Subsequent addition of Ca^{2+} in excess of EDTA did not return the spectrum to that of the Ca^{2+}-stabilized structure. Thus, the conformational change was for the most part irreversible.

Tryptophan fluorometry of Dsg1 was measured in various conditions (**Fig 6**). The upper graph in **Fig 6** shows hDsg1E in $TBS + Ca^{2+}$ (blue line), hDsg1E with 5 mM EDTA (red line), and hDsg1E with 5 mM EDTA and sequential addition of 10 mM $CaCl_2$ (green line). After addition of EDTA Dsg1E showed decreased intensity of fluorescence and this change was not reversed when Ca^{2+} was added back. These three curves, however, showed the same wavelength, 335 nm, of maximum emission, which suggest that hDsg1E is not completely denatured with EDTA, because completely denatured molecules usually have tryptophan exposed to water with a maximum emission at approximately 355 nm. In control experiments with tryptophan in $TBS + Ca^{2+}$, the addition of 5 mM EDTA and sequential addition of 10 mM $CaCl_2$ did not significantly change the fluorescent spectrum, suggesting that there was no quenching effect from EDTA or excess Ca^{2+} (data not shown). The middle graph of **Fig 6** shows hDsg1E in $TBS + Ca^{2+}$, pH 7.4 (blue line), Dsg1E in pH 3.5 (red line), and Dsg1E pretreated at pH 3.5 then neutralized to pH 7.0 (green line). In pH 3.5, Dsg1E showed decreased intensity of fluorescence and this change was not reversed with pH 7.4. Again, these three curves showed maximum emission at the same wavelength, approximately 335 nm, with striking resemblance to the tryptophan fluorescence spectra of hDsg1E treated with EDTA, suggesting the major effect of pH on conformation of Dsg1 may be mediated through loss of binding of Ca^{2+}, and that this effect is not reversible.

To show that both EDTA and low pH do not fully denature hDsg1E, we incubated it in 8 M urea (**Fig 6**, bottom graph). After 1 min, hDsg1E still showed little change compared with the molecule stabilized with Ca^{2+}. After 13 min, however, the spectrum showed a shift of the fluorescence peak to 347 nm (black line), consistent with more unfolding and exposure of tryptophan residues to water.

These data suggest that depletion of Ca^{2+} causes a mostly irreversible change in the conformation of Dsg1, but does not completely denature the molecule.

DISCUSSION

ETs show exquisite specificity for Dsg1. In neonatal mice and in the human disease, staphylococcal scalded skin syndrome, they circulate through the entire body, yet their major pathology is limited to the superficial epidermis, and this pathology is caused, as far as we know, by their proteolysis of only one peptide bond in one molecule, Dsg1. The crystal structure of ETA and ETB suggests that they are serine proteases that would cleave after a glutamic or aspartic amino acid (Cavarelli *et al*, 1997; Vath *et al*, 1997, 1999; Papageorgiou *et al*, 2000). Yet clearly ETs do not indiscriminately cleave substrates after these residues. We have shown that part of their specificity is contained in their ability to bind Dsg1 (Hanakawa *et al*, 2002). What accounts for this extremely focused specificity of binding and cleavage, however, has not been determined. In this study we show that this specificity is dependent on the calcium-stabilized conformation of Dsg1.

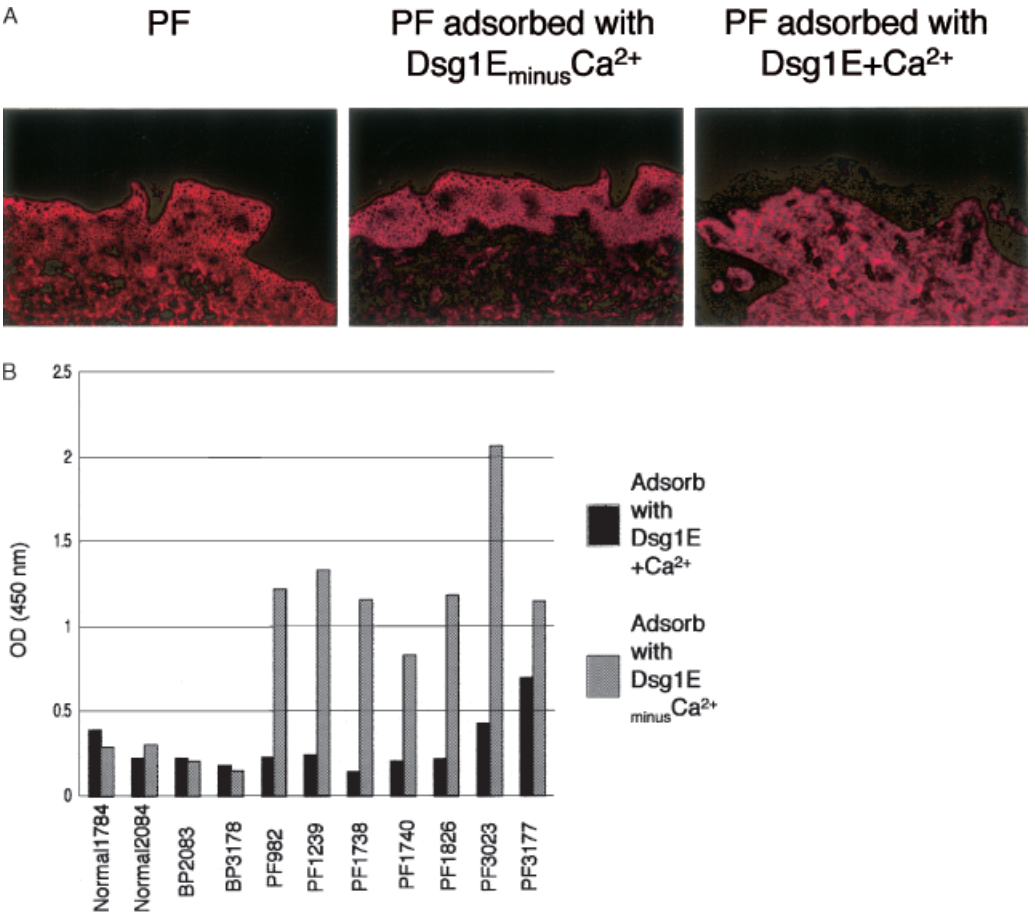

Figure 4. Indirect immunofluorescence staining and anti-Dsg1 ELISA of PF serum adsorbed with hDsg1E$_{minus}$Ca^{2+} or hDsg1E + Ca^{2+}. (*A*) PF serum was incubated with hDsg1E in TBS + Ca^{2+} or in TBS$_{minus}$Ca^{2+}, then used for indirect immuno-fluorescence staining of normal human skin. hDsg + Ca^{2+} adsorbed out cell surface antibodies, but hDsg1$_{minus}$Ca^{2+} did not. (*B*) Normal human sera, bullous pemphigoid patients' sera, and PF patients' sera were pretreated with hDsg1E qin TBS + Ca^{2+} or hDsg1E in TBS$_{minus}$Ca^{2+}, then used for ELISA assay against Dsg1. hDsg1E + Ca^{2+} showed significant increased adsorption compared with hDsg1E$_{minus}$Ca^{2+}.

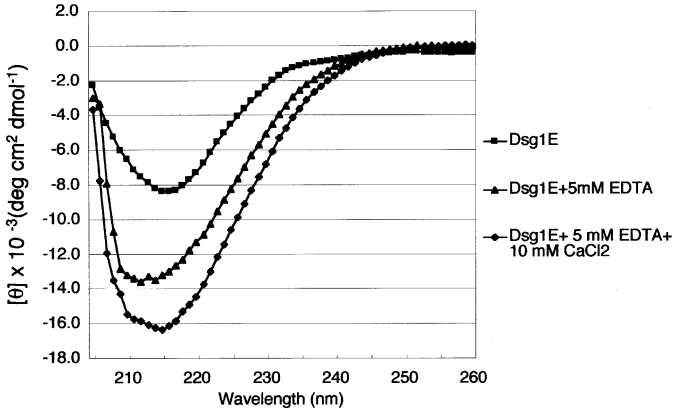

Figure 5. The spectrum of hDsg1E by circular dichroism with and without Ca^{2+}. The spectrum of hDsg1E in H_2O with 1 mM $CaCl_2$ (square), after treatment with 5 mM EDTA (triangle), then after adding back 10 mM $CaCl_2$ (diamond). EDTA resulted in a shift of the minimum ellipticity from approximately 215 nm to approximately 212 nm, with increased negativity. This change was not reversed when excess Ca^{2+} was added back.

The crystal structure of the extracellular domain of classical cadherins suggests that it forms a curved rod-like structure composed of the five extracellular subdomains that are stabilized by calcium-binding between these subdomains (Shapiro *et al*, 1995; Nagar *et al*, 1996; Tamura *et al*, 1998; Pertz *et al*, 1999; Boggon *et al*, 2002). The marked amino acid sequence homology of desmogleins and classical cadherins, including that of the calcium-binding domains, suggests that Dsg1, like these other cadherins, has a conformation stabilized by calcium. We confirmed this hypothesis by circular dichroism and tryptophan fluorometry. In the former, we showed a change in the conformation of the extracellular domain of Dsg1 when calcium was depleted, with a more negative ellipticity. This change was similar to that reported with the extracellular domain of E-cadherin as well as extracellular domains 1 and 2 of E-cadherin and Dsg2 (Pokutta *et al*, 1994; Koch *et al*, 1997b; Syed *et al*, 2002). Similarly, we showed a change in conformation of Dsg1 with tryptophan fluorescence, with a somewhat quenched fluorescence without a shift in peak fluorescence spectrum, when calcium was depleted. A similar finding has been reported for E-cadherin (Pokutta *et al*, 1994). Complete unfolding in the absence of calcium was not observed, however, as shown by the finding that urea induced a positive shift of the spectral peak, which indicates unfolding with more exposure of tryptophans to solvent. A similar shift with urea has been reported for Dsg2 (Syed *et al*, 2002).

As ETs cut in a calcium-binding domain of Dsg1, we hypothesized that the binding and cleavage of ETs might be dependent on the calcium-dependent structure of Dsg1. We confirmed this hypothesis by showing that heat and pH denaturation of Dsg1 prevented its cleavage by ETs as did the absence of calcium. Interestingly, the loss of susceptibility to proteolysis by ETs was mostly irreversible with calcium repletion, suggesting that the change in structure of Dsg1 induced by calcium depletion was not easily reversible simply by adding back calcium. This result is consistent with our circular dichroism and tryptophan fluorescence data. Unlike previous studies with E-cadherin and Dsg2 that suggest the change in conformation with calcium depletion is reversible when calcium is repleted (Pokutta *et al*, 1994; Koch *et al*, 1997b; Syed *et al*, 2002), our studies indicated that the conformational change was not totally reversible. Although the explanation for this discrepancy is not clear, it may reside in

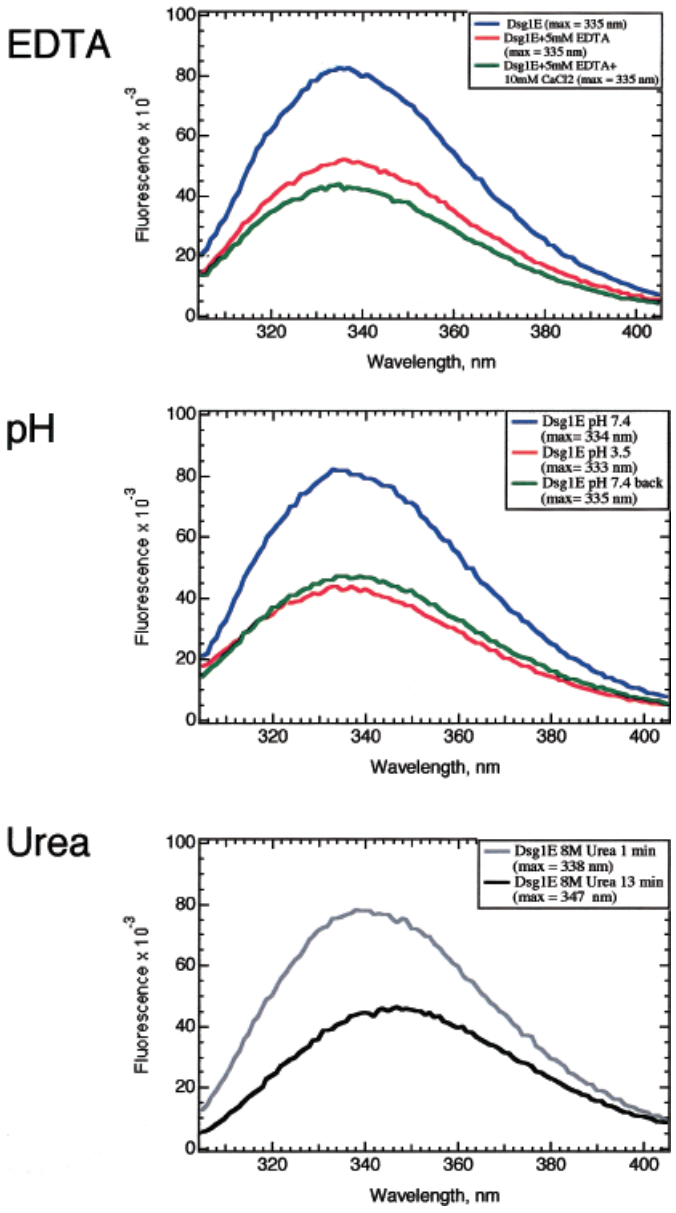

Figure 6. Tryptophan fluorescence of Dsg1. Tryptophan fluorescence spectra of hDsg1E treated with EDTA (upper graph), acid pH (middle graph), and 8 M Urea (lower graph) were measured with a spectrofluorometer at 25°C after excitation at 295 nm. Maximum tryptophan fluorescence intensity of hDsg1E in TBS + Ca^{2+} (blue line) was seen at 335 nm. When 5 mM of EDTA was added to hDsg1E (red line), the intensity was decreased but still peaked at 335 nm. Subsequent adding of excess $CaCl_2$ (green line) did not reverse this loss of intensity. These data suggest that chelation of calcium resulted in a change in conformation of Dsg1 that resulted in increased quenching of tryptophan fluorescence. Maximum tryptophan fluorescence intensity of hDsg1E at pH 7.4 (blue line) was reduce at pH 3.5 (red line), and not restored when pH was subsequently adjusted back to pH 7.0 (green line). These changes are similar to those seen with EDTA treatment. As opposed to the change only in intensity seen with EDTA and pH, 8 M urea caused also caused a in the wavelength of peak intensity from 338 nm at 1 min (gray line) to 247 nm at 13 min (black line). This shift implies more complete denaturation with urea than with loss of calcium or acidic pH.

inherent differences in Dsg1 compared with E-cadherin and Dsg2. In addition, our results showing that the ability of ETs to cleave Dsg1, which is lost with calcium depletion, is not regained with calcium repletion, are consistent with our spectroscopic findings that the conformation of Dsg1 is not restored. In the absence of calcium, Dsg1 may unfold or refold in such a way that new hydrophilic and hydrophobic interactions occur that are kinetically stable and not easily reversible with repletion of calcium.

The fact that ETs only cleave Dsg1 in its calcium-stabilized structure implies a very specific conformation interaction of ETs with Dsg1. This is of particular interest in light of several reported crystal structures of ETA and ETB from which it has been proposed that a specific substrate must interact with it in such a way that a particular peptide bond is rotated 180° in order to form properly the catalytic site (Cavarelli *et al*, 1997; Vath *et al*, 1997, 1999). The data presented here suggest that the specificity of ETs for Dsg1 in its calcium-stabilized conformation may allow a very specific fit, which could in turn result in a change in conformation of ETs and/or the correct alignment of ETs with its specific cleavage site.

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REFERENCES

Amagai M, Hashimoto T, Green KJ, Shimizu N, Nishikawa T: Antigen-specific immuoabsorption of pathogenic autoantibodies in pemphigus foliaceus. *J Invest Dermatol* 104:895–901, 1995a

Amagai M, Ishii K, Hashimoto T, Gamou S, Shimizu N, Nishikawa T: Conformational epitopes of pemphigus antigens (Dsg1 and Dsg3) are calcium dependent and glycosylation independent. *J Invest Dermatol* 105:243–247, 1995b

Amagai M, Matsuyoshi N, Wang ZH, Andl C, Stanley JR: Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. *Nature Med* 6:1275–1277, 2000

Amagai M, Yamaguchi T, Hanakawa Y, Nishifuji K, Sugai M, Stanley JR: Staphylococcal exfoliative toxin B specifically cleaves desmoglein 1. *J Invest Dermatol* 118:845–850, 2002

Angst BD, Marcozzi C, Magee AI: The cadherin superfamily. Diversity in form and function. *J Cell Sci* 114:629–641, 2001

Boggon TJ, Murray J, Chappuis-Flament S, Wong E, Gumbiner BM, Shapiro L: C-cadherin ectodomain structure and implications for cell adhesion mechanisms. *Science* 296:1308–1313, 2002

Cavarelli J, Prevost G, Bourguet W, *et al*: The structure of *Staphylococcus aureus* epidermolytic toxin A, an atypic serine protease, at 1.7A resolution. *Structure* 5:813–824, 1997

Chidgey M, Brakebusch C, Gustafsson E, *et al*: Mice lacking desmocollin 1 show epidermal fragility accompanied by barrier defects and abnormal differentiation. *J Cell Biol* 155:821–832, 2001

Chitaev NA, Troyanovsky SM: Direct Ca2 + -dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell–cell adhesion. *J Cell Biol* 138:193–201, 1997

Eyre RW, Stanley JR: Human autoantibodies against a desmosomal protein complex with a calcium-sensitive epitope are characteristic of pemphigus foliaceus patients. *J Exp Med* 165:1719–1724, 1987

Garrod D, Chidgey M, North A: Desmosomes: Differentiation, development, dynamics and disease. *Curr Opin Cell Biol* 8:670–678, 1996

Green KJ, Gaudry CA: Are desmosomes more than tethers for intermediate filaments? *Nature Rev Mol Cell Biol* 1:208–216, 2000

Hanakawa Y, Schechter N, Lin C, *et al*: Molecular mechanisms of blister formation in bullous impetigo and staphylococcal scalded skin syndrome. *J Clin Invest* 110:53–60, 2002

Hashimoto T, Kiyokawa C, Mori O, *et al*: Human desmocollin 1 (Dsc1) is an autoantigen for the subcorneal pustular dermatosis type of IgA pemphigus. *J Invest Dermatol* 109:127–131, 1997

Ishii K, Amagai M, Hall RP, Hashimoto T, *et al*: Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins. *J Immunol* 159:2010–2017, 1997

Kemler R, Ozawa M, Ringwald M: Calcium-dependent cell adhesion molecules. *Curr Opin Cell Biol* 1:892–897, 1989

Koch PJ, Mahoney MG, Ishikawa H, *et al*: Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. *J Cell Biol* 137:1091–1102, 1997a

Koch AW, Pokutta S, Lustig A, Engel J: Calcium binding and homoassociation of E-cadherin domains. *Biochemistry* 36:7697–7705, 1997b

Kondo I, Sakurai S, Sarai Y: New type of exfoliatin obtained from staphylococcal strains, belonging to phage groups other than group II, isolated from patients with impetigo and Ritter's disease. *Infect Immun* 10:851–861, 1974

Koulu L, Kusumi A, Steinberg MS, Klaus Kovtun V, Stanley JR: Human autoantibodies against a desmosomal core protein in pemphigus foliaceus. *J Exp Med* 160:1509–1518, 1984

Kowalczyk AP, Anderson JE, Borgwardt JE, Hashimoto T, Stanley JR, Green KJ: Pemphigus sera recognize conformationally sensitive epitopes in the amino-terminal region of desmoglein-1 (Dsg1). *J Invest Dermatol* 105:147–152, 1995

Ladhani S, Joannou CL, Lochrie DP, Evans RW, Poston SM: Clinical, microbial, and biochemical aspects of the exfoliative toxins causing staphylococcal scalded-skin syndrome. *Clin Microbiol Rev* 12:224–242, 1999

Mahoney MG, Wang Z, Rothenberger K, Koch PJ, Amagai M, Stanley JR: Explanation for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *J Clin Invest* 103:461–468, 1999

Marcozzi C, Burdett ID, Buxton RS, Magee AI: Coexpression of both types of desmosomal cadherin and plakoglobin confers strong intercellular adhesion. *J Cell Sci* 111:495–509, 1998

Melish ME, Glasgow LA: The staphylococcal scalded-skin syndrome. Development of an experimental model. *N Engl J Med* 282:1114–1119, 1970

Melish ME, Glasgow LA: Staphylococcal scalded skin syndrome: The expanded clinical syndrome. *J Pediatrics* 78:958–967, 1971

Melish ME, Glasgow LA, Turner MD: The staphylococcal scalded-skin syndrome. Isolation and partial characterization of the exfoliative toxin. *J Infect Dis* 125:129–140, 1972

Nagar B, Overduin M, Ikura M, Rini JM: Structural basis of calcium-induced E-cadherin rigidification and dimerization. *Nature* 380:360–364, 1996

Ozawa M, Engel J, Kemler R: Single amino acid substitutions in one Ca2+ binding site of uvomorulin abolish the adhesive function. *Cell* 63:1033–1038, 1990

Papageorgiou AC, Plano LR, Collins CM, Acharya KR: Structural similarities and differences in *Staphylococcus aureus* exfoliative toxins A and B as revealed by their crystal structures. *Protein Sci* 9:610–618, 2000

Pertz O, Bozic D, Koch AW, Fauser C, Brancaccio A, Engel J: A new crystal structure, Ca2+ dependence and mutational analysis reveal molecular details of E-cadherin homoassociation. *EMBO J* 18:1738–1747, 1999

Pokutta S, Herrenknecht K, Kemler R, Engel J: Conformational changes of the recombinant extracellular domain of E-cadherin upon calcium binding. *Eur J Biochem* 223:1019–1026, 1994

Ringwald M, Schuh R, Vestweber D, *et al*: The structure of cell adhesion molecule uvomorulin. Insights into the molecular mechanism of Ca2+-dependent cell adhesion. *EMBO J* 6:3647–3653, 1987

Shapiro L, Fannon AM, Kwong PD, *et al*: Structural basis of cell–cell adhesion by cadherins. *Nature* 374:327–337, 1995

Stanley JR, Koulu L, Klaus Kovtun V, Steinberg MS: A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J Immunol* 136:1227–1230, 1986

Steinberg MS, McNutt PM: Cadherins and their connections: Adhesion junctions have broader functions. *Curr Opin Cell Biol* 11:554–560, 1999

Syed SE, Trinnaman B, Martin S, Major S, Hutchinson J, Magee AI: Molecular interactions between desmosomal cadherins. *Biochem J* 362:317–327, 2002

Tamura K, Shan WS, Hendrickson WA, Colman DR, Shapiro L: Structure-function analysis of cell adhesion by neural (N-) cadherin. *Neuron* 20:1153–1163, 1998

Vath GM, Earhart CA, Rago JV, Kim MH, Bohach GA, Schlievert PM, Ohlendorf DH: The structure of the superantigen exfoliative toxin A suggests a novel regulation as a serine protease. *Biochemistry* 36:1559–1566, 1997

Vath GM, Earhart CA, Monie DD, Iandolo JJ, Schlievert PM, Ohlendorf DH: The crystal structure of exfoliative toxin B: A superantigen with enzymatic activity. *Biochemistry* 38:10239–10246, 1999

Wallis S, Lloyd S, Wise I, Ireland G, Fleming TP, Garrod D: The alpha isoform of protein kinase C is involved in signaling the response of desmosomes to wounding in cultured epithelial cells. *Mol Biol Cell* 11:1077–1092, 2000

Yamaguchi T, Nishifuji K, Sasaki M, *et al*: Identification of the *Staphylococcus aureus etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infect Immun* 70:5835–5845, 2002

Yasuda H, Kobayashi H, Hashimoto T, Itoh K, Yamane M, Nakamura J: Subcorneal pustular dermatosis type of IgA pemphigus: Demonstration of autoantibodies to desmocollin-1 and clinical review. *Br J Dermatol* 143:144–148, 2000